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## **Skeletal muscle differentiation drives a dramatic downregulation of RNA polymerase III activity and differential expression of Polr3g isoforms**

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### **Abstract**

Gene regulatory networks underpinning skeletal muscle determination and differentiation have been extensively investigated, providing molecular insights into how cell lineages are established during development. These studies have exclusively focused on the transcriptome downstream of RNA polymerase II (Pol II). RNA polymerase III (Pol III) drives the production of tRNAs and other small RNAs essential for the flow of genetic information from gene to protein and we have found that a specific isoform of a subunit unique to Pol III is expressed early in the myogenic lineage. This points to the possibility that additional regulatory networks exist to control the production of Pol III transcripts during skeletal muscle differentiation. We describe the differential expression of Polr3g and its alternate isoform Polr3gL during embryonic development and using a custom tRNA microarray, we demonstrate their distinct activity on the synthesis of tRNA isoacceptors. We show that Pol III dependent transcripts are dramatically down-regulated during the differentiation of skeletal muscle, as are mRNAs coding for Pol III associated proteins Brf1 and Brf2, while Polr3gL is up-regulated alongside contractile protein genes. Forcing Polr3g expression in this context results in a partial reversal of myogenic differentiation.

### **Introduction**

Differential gene expression drives the establishment of distinct cell lineages during development and the transcriptional regulation of protein coding genes during this process has been widely studied. For instance, the establishment of the myogenic cell lineage requires a set of bHLH transcription factors known collectively as myogenic regulatory factors (MRFs) that include myoD, myf5, myogenin, and MRF4 (Pownall et al., 2002). These factors are central to a gene regulatory network (GRN) that first promotes the proliferation of a progenitor population with myogenic potential and later activates the coordinated transcription of skeletal muscle specific genes such as those that code for contractile proteins (Fong and Tapscott, 2013; Moncaut et al., 2013). While these studies have focused only on transcription driven by RNA polymerase II (Pol II), the regulation of RNA polymerase III (Pol III) activity during myogenesis remains largely unprobed in this context.

Among other targets, such as U6 and 5S RNAs, Pol III synthesises the tRNAs that decode the Pol II mRNAs during protein synthesis; in this way, the interaction between Pol II and Pol III products enables the flow of genetic information. In bacteria, differential expression of tRNA isoacceptors (that recognise specific codons) is a mechanism used to control translation of specific proteins (Kane, 1995). Quantitative analysis of RNA-seq and ChIP-seq data investigating the expression of mRNA and tRNA genes in different lineages has revealed a remarkable correlation between the codon demands of protein coding genes and the expression of the corresponding anticodon tRNA genes (Gingold et al., 2014) (Dittmar et al., 2006). The observation that codon usage changes during development, while a stable pool of corresponding anticodon tRNA isoacceptors is maintained, indicates that

regulatory mechanisms must exist to stabilise the 'mRNA-tRNA' interface (Schmitt et al., 2014) (Rudolph et al., 2016). The extent of Pol II /Pol III coordination, that is, the balance of codon demand to tRNA supply, is thought to contribute to cellular fitness (Gingold and Pilpel, 2011). For instance, the transcriptomes of proliferative and differentiated cells have been shown to actively balance the synthesis of specific tRNAs with the codon usage of mRNAs expressed in each state (Gingold et al., 2014).

In contrast to Pol I and Pol II, RNA polymerase III is present in two different isoforms, one of which is enriched in proliferative cells, including stem cells and some tumour cell lines (Jin et al., 2016; Wong et al., 2011). The distinct isoforms of Pol III are distinguished by the presence of the small subunit Polr3g (RPC32 $\alpha$ ) or Polr3gL (RPC32 $\beta$ ) coded for by different genes (Haurie et al., 2010). The POLR3G (g or gL) subunit is unique to Pol III with no corresponding subunit in RNA Polymerase I or Pol II. Polr3g isoforms are part of a dissociable tripartite sub-complex with PolR3C (RPC62) and PolR3F(RPC39) that recruits Pol III to target genes (Wang and Roeder, 1997) forming a bridge between TFIIIB and the Pol III core enzyme (Boissier et al., 2015). The Polr3g isoform is expressed at high levels in proliferative cells and its expression decreases during differentiation (Haurie et al., 2010; Wong et al., 2011). Consistent with its association with proliferation, Polr3g expression increases during cellular transformation in cancer and its overexpression can elicit anchorage independent growth. In contrast, Polr3gL expression was not changed during differentiation or transformation (Haurie et al., 2010). Polr3g is expressed in undifferentiated human embryonic stem (ES) cells and knocking-down Polr3g decreases the expression of pluripotency genes Oct4, Nanog and Sox2 with a corresponding increase in differentiation markers, pointing to a role for Polr3g in maintaining pluripotency (Wong et al., 2011). miR-1305 has recently been implicated in restricting Polr3g during differentiation of pluripotent cells (Jin et al., 2016), supporting the notion that Polr3g is a pluripotency factor.

In this study, we describe the expression of *POLR3G* genes during the development of *Xenopus tropicalis* and show that the gene product for Polr3g is present as a maternal transcript and protein, and that it is expressed zygotically in cells early in the myogenic lineage. *Polr3gL* is only transcribed zygotically and more widely, including in differentiating muscle. We use a custom microarray to determine the effect of over expressing Polr3g and Polr3gL on the synthesis of tRNA isoacceptors and find the output is different depending on isoform expressed and stage analysed. We drive skeletal muscle differentiation in an explant model and find a dramatic down regulation of RNA Pol III activity associated with increased expression of *Polr3gL*. Together our results suggest that the differential expression of these two distinct isoforms of Pol III plays a role in the progressive establishment of cell fate.

## Methods

### *Embryological manipulations*

*X.tropicalis* embryos obtained by *in vitro* fertilisation were microinjected at the one or two cell stage with synthetic mRNA (specific doses are indicated in results and figure legends). Embryos were cultured in 5% Modified Ringers Saline until the stage indicated (Nieuwkoop and Faber, 1967). For animal cap explants, tungsten needles were used to dissect the animal pole region at late blastula

stage (Nieuwkoop and Farber (NF) Stage 8-9) that were cultured until tailbud stage (NF25). For dorsal dissections, stage 25 embryos were removed from their vitelline membranes and allowed to rest briefly before using mounted tungsten needles to dissect the head and ventral and dorsal sections. For collection, animal caps and dissected sections were snap-frozen on dry ice and stored at -80°C until use.

#### *Cloning and overexpression experiments*

Transcripts coding for *X.tropicalis* Polr3g (XM\_002942427) and Polr3gL (XM\_012967920) were identified in NCBI databases. To determine that the annotated sequences were correctly identified as Polr3g and Polr3gL in *Xenopus*, Polr3g and Polr3gL protein sequences for multiple vertebrate model organisms were identified in NCBI and a phylogenetic tree constructed in MegAlign showed that the protein sequence for *X.tropicalis* Polr3g and Polr3gL clustered with the corresponding protein in other species (data not shown but extensive phylogeny has been done on these genes (Renaud et al., 2014)). To generate probes for expression analyses of Polr3g and Polr3gL in *X.tropicalis* during development, primers were designed to amplify sequences from unique regions of Polr3g and Polr3gL using cDNA derived from *X.tropicalis* embryos; these products were cloned into Promega pGEM T-easy. The same primers were also used for semi-quantitative reverse transcriptase (RT)-PCR analysis. The plasmids were sequenced to confirm identity and determine the orientation of the insert. Antisense cRNAs were transcribed incorporating DIG-UTP (DIG RNA labelling mix Roche). For synthetic mRNA injection, the full length sequence of Polr3g and Polr3gL was amplified from cDNA derived from mixed staged *X.tropicalis* embryos, and directionally cloned into pCS2<sup>+</sup> incorporating an HA tag at the amino terminus. The plasmids were linearised and transcribed using Ambion MessageMachine. Synthetic mRNAs coding for FGF4 and Wnt8 were generated and injected as in (Burks et al., 2009).

#### *Whole-mount in situ hybridization*

*In situ* hybridization was carried out according to Harland (Harland, 1991) with some modifications. Vitelline membranes were removed manually from *X.tropicalis* embryos using forceps. Embryos were fixed in MEMFA (0.1M MOPS pH7.4, 2mM EGTA, 1mM MgSO<sub>4</sub>, 3.7% formaldehyde) for one hour and stored in 100% methanol at -20°C until use. Embryos were rehydrated through a series of washes of a gradient of methanol/PBS-Tween (75% methanol/ 25% PBST, 50% methanol/ 50% PBST, 100% PBST and permeabilised with 10ug/ml Proteinase K (Roche), treated with triethanolamine/acetic anhydride, and washed extensively in PBST before refixing in 10% formaldehyde. Embryos were then transferred into 50% PBSAT/ 50% hybridization buffer (50% formamide, 5X SSC, 1mg/ml total yeast RNA, 100µg/ml heparin, 1X Denharts, 0.1% Tween-20, 0.1% CHAPS, 10mM EDTA) at 60°C for 10 minutes. The solution was replaced with 100% hybridization buffer and embryos were prehybridised for 2 hours at 60°C. Embryos were then transferred into 1ml hybridization buffer + 2-3µl DIG probe and rocked overnight at 60°C. The following day, embryos were washed at 60°C in 2X SSC + 0.1% Tween-20, then 0.2X SSC + 0.1% Tween-20, before blocking at room temperature in Maleic Acid Buffer (MAB: 100mM maleic acid, 150mM NaCl, 0.1% Tween-20 pH7.8) + 20% heat treated lamb serum + 2% Roche Blocking Reagent for two hours. This was replaced with fresh blocking solution containing a 1:2000 dilution of affinity purified anti-DIG fragments coupled to alkaline phosphatase (Roche) and rolled overnight at 4°C. Embryos were washed three times for one hour in MAB before adding the AP substrate BM Purple™ staining

solution (Roche) and left at room temperature until colour developed. Embryos were fixed in 10% formalin and photographed.

#### *Western blotting*

For analysis of protein expression both endogenous and overexpressed, 20-25 *X.tropicalis* embryos were collected and homogenised in 30µl PhosphoSafe™ Extraction Reagent by pipetting. 5µl of 7X cOmplete™, Mini EDTA-free Protease Inhibitor cocktail (Roche) was added to the mix and embryos were immediately incubated at -80°C for 10 minutes. Samples were centrifuged at 13,000rpm for 20 minutes, the resulting supernatants were collected into new tubes and boiled at 95°C for 5 minutes in sample buffer before loading onto 15% Acrylamide SDS PAGE gels. For detecting endogenous Polr3g a custom antibody (Eurogentec) was raised against the epitope EIEAERKLQREW, affinity purified, and used 1:10,000 in 5% milk in PBST. Antibodies to GAPDH (Sigma), and HA (Sigma) were used under the same conditions at 1:10,000 and 1:1000 respectively.

#### *Total RNA extraction*

For extraction of total RNA from tissue samples, 10-15 embryos/20 explants were snap frozen using dry ice. Frozen samples were homogenised in 1ml Tri-Reagent (Sigma), centrifuged 10 minutes at 13,000rpm to remove yolk and transferred to a new tube. Phase separation with the addition of 200µl chloroform and centrifugation at 13,000rpm, was followed by isopropanol precipitation of the aqueous phase. Samples were centrifuged at 13,000rpm for 10 minutes to pellet the RNA, which was resuspended in 40µl of nuclease free water. RNA was then DNase and purified using the Zymo-Spin™ Clean and Concentrator™ Kit as per the manufacturer's instructions to ensure inclusion of small RNAs.

#### *qPCR*

cDNA was synthesised using 1µg of RNA and Superscript IV (Thermo-Fisher) as per manufacturer's instructions with random hexamers. Primers were designed using Integrated DNA Technologies PrimerQuest tool. Products were designed to be between 75 and 110 base pairs in length and primers were designed across exon junctions to prevent amplification of any residual genomic DNA (a table listing all primers is provided in the supplementary data). For qPCR, Fast SYBR Green was used in triplicate for three biological repeats. Average Ct was normalized to the housekeeping gene *Dicer* and pairwise t-tests were carried out comparing the mean relative expression for control and experimental sets (siblings) for each gene. When more than two conditions were compared, ANOVA analysis was carried out. Graphs were constructed in GraphPad Prism5. Error bars represent SEM, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ .

#### *Microarray design and analysis*

To analyse global tRNA expression dynamics in *Xenopus*, a custom microarray was designed to include probes for all tRNA isoacceptor families. Five 60mer probes were designed for each tRNA isoacceptor family passing quality checks using Agilent eArray software. Additional manual probes were designed for 5', 3' and middle sections of isoacceptor family sequences not included in the Agilent design. As a control for specificity, sequences for CHO and human tRNA isoacceptor families



were also manually designed. A total of 2058 probes were selected for Agilent's standard 8X15K array design; in order to fill the array the probes were replicated 7 times. RNA was extracted from embryos as described above and quality control was carried out using the Agilent Bioanalyzer 2000 to determine RNA Integrity Numbers (RINs between 7.4-7.9). cDNA was labelled with Cy3, hybridised on the array for 17 hours at 65°C, washed and scanned to create a high resolution TIFF file for processing of expression data. The results from our microarray analyses have been deposited on ArrayExpress ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) accession numbers are: E-Mtab-7735 (NF stage 9); E-Mtab-7736 (NF stage 25); and E-Mtab-7752 (FGF/WNT).

GraphPad Prism5 software was used for statistical tests (paired t-tests) on the mean fold change for each isoacceptor family and each amino acid isotype for paired samples, and to construct graphs of mean fold changes using a threshold of 1.2 and 0.8 for up/downregulation of transcription of tRNA families. Graphs include error bars indicating SEM and \* indicates the level of significance from the statistical analyses after Bonferroni Correction. To determine 'codon usage scores', the number of times a codon appears in the coding sequence of all mRNAs included on the microarray was calculated and multiplied by the intensity value for that mRNA. A sum of these values was calculated for each mRNA with an intensity value of above 10, and graphed against tRNA expression values measured as their intensity values on the array. Correlation was calculated using Spearman's Rho assessment for monotonic relationship between variables. Correlation coefficients between 0 and 1 were calculated and p-values presented.

## Results

### *Expression of POLR3G isoforms*

Temporal profiling of *Polr3g* and *Polr3gL* expression in *X.tropicalis* reveals a complementary expression pattern where high levels of *Polr3g* transcripts are detected in the early embryo, while *Polr3gL* is not detected until later (Figure 1). During vertebrate development, the earliest cleavage stages are characterised by rapid cell division and are supported by stored maternal mRNA and proteins while the zygotic genome is silent until a point called the midblastula transition (MBT, between NF stage 8 and 9) when the cell cycle lengthens and zygotic transcription begins. We have found that *Polr3g* transcripts and protein are present prior to MBT and later fall to a lower level, while in contrast, *Polr3gL* transcription is activated after MBT and increases during early development. Figure 1A shows a semi-quantitative RT-PCR where *Polr3g* expression is highest maternally, during early cleavage, prior to MBT. After the activation of embryonic transcription, *Polr3g* expression decreases rapidly and *Polr3gL* expression is activated. Western blotting demonstrates *Polr3g* protein expression is highest in embryos prior to MBT, however, a low level of protein persists into later developmental stages suggesting that *Polr3g* protein is relatively stable *in vivo* (Figure 1B). Interrogating an RNA-seq database available online ([www.xenbase.org](http://www.xenbase.org)) provides additional data consistent with our findings that *Polr3g* and *Polr3gL* have a complementary expression during *Xenopus* development. (Figure 1C (Owens et al., 2016)).

To determine the spatial localisation of *Polr3g* isoforms during development, we used *in situ* hybridization to analyse endogenous gene expression in a stage series of *X.tropicalis* embryos (Figure 2 and 3; also see supplementary Figures 1 and 2). We find a remarkable, specific expression of

zygotic *Polr3g* in the early somites (Figure 2A-C), the source of all skeletal muscle in the vertebrate body. *Polr3g* is expressed in the muscle forming region from neurula stages while the analysis of later embryos reveal that this expression is transient as by late tailbud stages *Polr3g* is no longer expressed in the somites, but rather it is expressed in the pronephros and the eye (supplemental data). In contrast, *Polr3gL* is not expressed in the somites at this stage, but rather in the eye, neural tube and branchial arches (Figure 3A). At later stages, *Polr3gL* continues to be expressed at high levels in the head and the ventral blood island, and in the posterior somites (Figure 3C and D). To further assess the specificity of *Polr3g* isoform expression in the somites, RNA was extracted from dissected sections of NF stage 23 (Head, Ventral and Dorsal). Quantitative RT PCR analysis (qPCR) confirmed the enrichments of *Polr3g* transcripts in the dorsal, somitic region of the early embryo (Figure 2D), while at this same stage *Polr3gL* transcripts are found both anteriorly and dorsally (Figure 3B), consistent with its expression in the head and neural tube at this stage.

#### *Measuring Pol III activity*

Among other targets, RNA polymerase III (Pol III) transcribes the tRNA genes, and there are challenges in analysing the transcription of these small, highly structured RNAs. Antibodies against Pol III have been used for ChIP-seq, identifying different tRNA genes that are active in different cells (Schmitt et al., 2014). These studies were carried out in mammals that have 400-600 tRNA genes compared to the 2638 tRNA genes in the *X.tropicalis* genome (<http://gtrnadb.ucsc.edu/>). The extensive repetitive nature of almost identical tRNA sequences in *Xenopus* would make the computational analysis downstream of ChIP-seq problematic. We therefore opted to directly assess the tRNA transcriptome, which has challenges of its own because of the highly-modified nature of tRNAs. Reverse transcriptase based protocols that rely on the priming and extension of the first strand cDNA are not very efficient on tRNAs; for instance, high throughput sequencing methods only work when samples are pre-treated with demethylases for DM-tRNA-seq (Zheng et al., 2015). We observed these phenomena when preparing cDNA from a stage series of early *Xenopus* embryos: using RT-PCR, we detected no tRNAs in cDNA prepared from samples prior to mid-blastula transition (MBT), the point when the zygotic genome initiates transcription (Supplementary Figure 3A). Clearly, tRNAs are present in early frog embryos and oocytes that readily produce protein from injected mRNAs, and this artefact is due to a failure of cDNA synthesis from highly structured tRNAs. The denaturing conditions of Northern blotting reveals the high expression of the mature tRNA transcripts for Tyrosine throughout the stages examined (73nt) (Supplementary Figure 3B). The newly synthesised transcripts are discernible as unprocessed 103nt and 86nt transcripts and detected after MBT; this is the same time point when it is possible to detect tRNA by RT-PCR. We conclude that newly transcribed tRNAs that are activated in *Xenopus* at MBT (Newport and Kirschner, 1982b) and native tRNA transcripts from active transcription can be analysed using cDNA-based methods.

#### *Polr3g has differential effects on transcription of tRNA isoacceptors upregulating a subset of mRNAs*

Large-scale analysis of transcriptional changes as a response to *Polr3g* overexpression was undertaken using a custom microarray (Agilent) that includes probes for tRNAs and other RNA polymerase III targets, as well as selected mRNAs. Multiple tRNA anticodon families (isoacceptors) encode each amino acid isotype and the different isoacceptor families reflect the redundancies in the translational code. For instance, methionine has only a single anticodon tRNA Met<sup>CAT</sup>, while



arginine has six different and partially redundant tRNA anticodons recognised by a family of isoacceptors: tRNA Arg<sup>ACG</sup>, Arg<sup>GCG</sup>, Arg<sup>CCG</sup>, Arg<sup>TCG</sup>, Arg<sup>CCT</sup> and Arg<sup>TCT</sup>. In *Xenopus* there are 54 isoacceptor families encoding the 20 standard amino acid isotypes and each anticodon isoacceptor is encoded by multiple tRNA genes. In *Xenopus tropicalis*, it is estimated that there are 2638 tRNA genes (Chan and Lowe, 2016) where gene duplication means that the expression analysis of individual tRNA genes is impossible using hybridization or primer-based methods. Therefore our data on the regulation of tRNA expression is analysed at the level of the isoacceptor family (codon specific) rather than at the level of individual tRNA genes.

To determine whether Polr3g overexpression has an effect on tRNA expression during development, 2ng of synthetic mRNA coding for Polr3g was injected into *X.tropicalis* embryos at the 2-cell stage cultured until just after MBT (NF stage 9). RNA was extracted from three biological repeats of Polr3g injected and paired sibling control embryos, and labelled cDNA was hybridised to the array. Analysis of both mean fold change and differences between the means of control and experimental samples were carried out for tRNAs at the level of isoacceptor and amino acid isotype and summarised in Figure 4. Fold changes of the mean normalized expression for each anticodon family are shown with fold change thresholds of 0.8 and 1.2 were used to define families as downregulated or upregulated respectively. Polr3g injected samples showed significant differences in tRNA expression levels for many isoacceptor families when compared to control samples. Overall more isoacceptor families are upregulated than are downregulated as a result of Polr3g overexpression. In addition, Polr3g activity in blastula embryos results in distinct changes in expression of some isoacceptor families (Figure 4A). For instance, tRNA<sup>Gln</sup> has two isoacceptor families present at Stage 9 and while Gln<sup>CTG</sup> is significantly upregulated by overexpression of Polr3g, expression of Gln<sup>TTG</sup> is decreased to 0.64 relative to control expression levels. A similar change in expression occurs in tRNA<sup>Thr</sup> families. tRNA<sup>Ser</sup> isoacceptor families show variable regulation by Polr3g; Ser<sup>AGA</sup> and Ser<sup>GCA</sup> families show no change in expression, Ser<sup>GCT</sup> is significantly upregulated and Ser<sup>TGA</sup> is significantly down-regulated in embryos overexpressing Polr3g. We find that complementary changes in expression of other tRNA isoacceptor genes equilibrates the overall level of tRNAs for each amino acid isotype (data not shown), consistent with a previous study (Schmitt et al., 2014). Interestingly, other RNA polymerase III genes (both 5S rRNAs, 7SK and 7SL RNAs) were significantly downregulated in samples overexpressing Polr3g compared with control embryos, while another Pol III target, U6 RNA, appeared to show some level of upregulation (Figure 4B). We conclude that Polr3g activity can differentially regulate a subset of tRNA isoacceptor families and other Pol III genes during early development and can selectively activate, and occasionally repress transcription.

Several mRNAs selected for analysis are expressed at higher levels in embryos overexpressing Polr3g compared to sibling controls. mRNAs shown to be significantly upregulated include a number of key regulators of early embryonic patterning (Figure 4C): the BMP antagonist *chordin* (*chrd*), the forkhead box transcriptional repressor *foxd5*; the bHLH transcriptional repressor *id3*; *wnt8a*, a ligand involved in ventral mesoderm patterning; the Spemann Organiser transcription factor (*gsc*); a mesodermal factor, *eomesodermin* (*eomes*); theTGf $\beta$  ligand *nodal* (*xnr4*); and the nodal inhibitor, *lefty*. The mechanism by which this Pol III subunit affects Pol II gene transcription is unknown, but similar observations where overexpression of Polr3g results in changes in mRNAs have been described elsewhere (Lund et al., 2017).

### *Differential regulation of tRNAs by Polr3g and Polr3gL*

Given the specific expression of Polr3g in the somites at early tailbud stages, and Polr3gL in later myogenesis, we wanted to determine the effect of overexpression of Polr3g as compared to Polr3gL on the synthesis of tRNAs in the muscle lineage. To do this, embryos were injected at the 2-cell stage with 2ng of synthetic mRNA coding for either Polr3g or Polr3gL and the dorsal regions dissected at Stage 25 for analysis. At this stage, the somites present in the dorsal region express MyoD and these cells are committed to the skeletal muscle lineage. Expression constructs included an HA tag to monitor levels of protein resulting from overexpression and western blot analysis shows that equal amounts of Polr3g and Polr3gL were present in all three biological replicates (Figure 5A). Dorsal regions were collected as illustrated in Figure 5B and qRT-PCR analysis for expression of MyoD confirmed accurate dissections (data not shown). Labelled cDNA was hybridised to custom microarrays as described in methods.

In contrast to the ability of Polr3g to activate tRNA synthesis at blastula stages, Polr3g shows very little activity in this more differentiated tissue (Figure 5C). Polr3gL, in contrast, represses transcription of tRNAs when overexpressed in dorsal lineages (Figure 5D). Figure 5C and 5D summarize the expression of tRNA isoacceptor families expressed above a threshold values of 10 in response to overexpression of Polr3g and Polr3gL. Polr3g overexpression results very few significant changes to tRNA synthesis. Polr3gL overexpression downregulates some isoacceptor families; for instance, all tRNA<sup>Ala</sup> families, Arg<sup>TCG</sup>, Ile<sup>AAT</sup>, Leu<sup>CAA</sup>, Leu<sup>AAG</sup> and Ser<sup>GCT</sup>. Overexpression of Polr3gL results in significant upregulation of Tyr<sup>GTA</sup>, and no significant changes to tRNA<sup>Val</sup> families, while Polr3g overexpression results in no change in Tyr<sup>GTA</sup> but significant upregulation of Val<sup>IAC</sup> and Val<sup>CAC</sup> at this stage.

In order to generate an overview of any distinct transcriptional effects of Polr3g and Polr3gL overexpression in this tissue, a scatterplot of these data was used to compare normalised expression of tRNA isoacceptors in samples overexpressing each factor. In Figure 5E, the X-axis shows expression of tRNA isoacceptors in Polr3g samples and the Y-axis shows their expression in Polr3gL samples. The dashed lines delineate a 2-fold change in expression and all points lie within these margins suggesting no significant difference between the samples. The overall effect of overexpression of Polr3gL at this stage is to down regulate tRNA synthesis, while Polr3g shows little activity at this stage (Figure 5E).

Our expression analysis indicates that Polr3gL is expressed during myogenic differentiation, replacing the transient expression of Polr3g, suggesting Polr3g and Polr3gL have distinct activities during myogenic differentiation. We investigated whether the overexpression of Polr3g or Polr3gL leads to changes in the expression of any myogenic genes in St25 dorsal regions and found that several contractile protein genes were found to be significantly upregulated in dorsal regions overexpressing Polr3gL, while unaffected by Polr3g (Figure 5F).

### *Correlation of tRNA abundance with codon usage in skeletal muscle specific genes*

Previous studies have correlated tRNA expression with mRNA codon usage in specific tissues (Dittmar et al., 2006; Gingold et al., 2014). To assess this relationship *in vivo* during skeletal muscle development, Codon Usage Estimate values were calculated by multiplying codon usage of mRNAs by their gene expression value and this value is plotted on the x-axis. For blastula stage 9, codon

usage was calculated for all transcribed mRNAs while for stage 25 explants, we focussed on the contractile protein mRNAs that were included on the arrays. Codon usage is compared to tRNA anticodon isoacceptor intensity using values from the array as a measure of tRNA expression (y-axis) (Supplemental Figure 4). Spearman's correlation analysis was carried out on the microarray expression data from control samples at Stage 9 for all tRNAs and expressed mRNAs, not assuming a linear relationship. The scatterplots shown in supplemental data indicate a weak correlation (0.175) between tRNA expression and mRNA codon use at stage 9, when cells remain in an undifferentiated state. At NF Stage 25, a time when muscle genes are activated in these dorsal samples, the data indicate a moderate correlation (0.349), suggesting that tRNA transcription is increasingly regulated to match mRNA codon usage during the establishment of the skeletal muscle lineage. This is consistent with the theory that regulatory mechanisms exist to stabilise the 'mRNA-tRNA' interface suggested by other researchers (Dittmar et al., 2006; Gingold et al., 2014; Hentzen et al., 1981).

#### *Pol III activity is downregulated during myogenic differentiation*

To test the possibility that there is a muscle specific tRNA profile in *Xenopus*, we exploited an explant model of myogenic differentiation. Overexpression of FGF4 alone in animal hemisphere explants is sufficient to induce a change in cell fate such that the tissues develops as largely ventral-type mesoderm (Slack et al., 1987). The induction of dorsal mesoderm (and muscle in particular) is enhanced through the co-injection of Fgf4 with Wnt8 (Burks et al., 2009). To test the existence of a myogenic-specific tRNA profile, this model of myogenic differentiation in animal caps was analysed by microarray.

Embryos were injected in the animal hemisphere of both blastomeres at the two-cell stage with synthetic mRNA coding for FGF4 (5pg) and Wnt8 (50pg). Explants from injected and sibling control embryos were taken at blastula stage 8 and cultures until NF stage 25 for RNA extraction. Sibling explants were cultured until NF stage 40 and the differentiation of skeletal muscle was confirmed by histology (not shown). Three biological repeats comprise 15 explants for each experimental and paired control sample were used to generate labelled cDNA and hybridised to our custom tRNA microarray. Strikingly, when compared to controls, all tRNA isoacceptor families detected on the microarray showed a significant reduction in response to Fgf4 and Wnt8 signalling (Figure 6A). In addition, all other RNA Polymerase III target genes were also significantly downregulated in experimental samples, indicating an overall down-regulation of RNA Polymerase III transcriptional activity in response to these myogenic inducing signals. Pol III activity was also found to be downregulated *in vivo* during differentiation, as the stage 25 dorsal explants show reduced transcription of tRNAs compared to blastula embryos (Supplemental Figure 5).

In contrast, the expression of contractile protein genes and the myogenic regulatory factor (MRF) genes are dramatically upregulated in Fgf4/Wnt8 caps (Figure 6B and C). Notably, the embryonic isoform of  $\alpha$ -actin, *actc1*, was upregulated indicating that the muscle lineages induced were in the early stages of differentiation. Analysis of MRF gene expression shows that the early regulator *Myf5* was upregulated in response to Fgf4 + Wnt8 with a mean fold change of 69.6, as was *MyoD* (fold change of 23.9). *Mrf4* and *Myogenin*, factors more associated with terminal differentiation, were less highly upregulated (fold change 12.4 and 6.4 respectively). These data are consistent with gene expression in cells early in the process of muscle lineage differentiation.

Genes coding for cell cycle associated proteins (*Ccne1*, *Ccne2*, *Mycl* and *Mycn*) were also upregulated in response to Fgf4 + Wnt8, and the expression of the cell cycle inhibitor *p27kip1* was significantly downregulated (Figure 6D). These data indicate that our myogenic explants retain cells that are proliferative, however, markers of pluripotency such as *Klf4*, *Klf6*, *Myc* and *Aurka* are either unchanged or down-regulated in these same samples. This suggests a loss of pluripotency during lineage determination and differentiation; indeed, determined myoblasts expressing *Myf5* and *MyoD* are still proliferative despite their commitment to a myogenic fate (Emerson, 1990; Pownall et al., 2002).

#### *Down-regulation of the Polymerase III transcription machinery during differentiation*

To determine a possible mechanism for the downregulation of RNA Polymerase III transcription in response to myogenic inducing signals, we analysed the expression levels of Pol III subunits and associated transcriptional machinery in response to FGF/Wnt (Figure 6E). Notably, the components of the TFIIIB complex, *Brf1* and *Bdp1*, are significantly downregulated in our experimental samples. These factors are known to be important for Pol III transcription initiation and have been implicated in the regulation of RNA Polymerase III transcription during differentiation (Athineos et al., 2010). The majority of RNA polymerase III subunits are downregulated in response to the initiation of muscle differentiation, with the exception of *Polr3A* and *Polr3C* (unchanged), and *Polr3K* and *Polr3gL* (increased) (Figure 6E). *Polr3gL* is associated with differentiation, and the upregulation of this isoform in response to muscle differentiation induced by FGF/Wnt is expected. *Polr3g* in contrast is not induced in this model of myogenesis despite its specific, transient expression in the early somites.

#### *Polr3g overexpression can reverse some aspects of myogenic differentiation*

*Polr3g* is associated with proliferative pluripotent cells and its expression is unchanged in our explant model of myogenic differentiation, however we nonetheless find elevated expression of some markers of proliferation. Our interpretation is that the population of proliferative cells in FGF/Wnt explants are no longer pluripotent, but rather are committed to the skeletal muscle cell lineage. The presence of *Polr3g* in early somites (Figure 2) suggests an early role in the muscle lineage, so we tested the ability of *Polr3g* to reverse the effects of myogenic induction on transcription by injecting mRNA coding for *Polr3g* in combination with FGF4 and Wnt8. We used qPCR to analyse a set of tRNAs isoacceptors and mRNAs coding for muscle proteins. Figure 7 shows the relative fold changes of expression of experimental versus paired control explants. Our data indicate that *Polr3g* overexpression is not sufficient to rescue the downregulation of a set of tRNA isoacceptors (Figure 7A) that is also observed in the microarray analysis in response to FGF/Wnt induction (Figure 6A). Surprisingly, overexpression of *Polr3g* did result in the downregulation of the FGF/Wnt induction of skeletal muscle differentiation markers (Figure 7B). Control explants do not express contractile protein genes, so the fold increase observed in FGF/Wnt treated explants is very large (values ranging from an average of 1600 to 125,000 fold increase) and we set these values to 1. When *Polr3g* is co-expressed with FGF4 and Wnt8, the explants show reduced expression of these differentiation genes. *actc1* is decreased to 14%, *act3* is decreased to 7% and *myh4* is decreased to 15% of the levels of gene expression activated by FGF4 and Wnt8. This finding that *Polr3g* expression is at least in part incompatible with cell differentiation is consistent with a previous study in ESCs showing that *Polr3g* overexpression results in a resistance of ESCs to respond to

differentiation by retinoic acid (Wong et al., 2011). We show here that Polr3g is not sufficient to rescue differentiation-induced restriction of Pol III transcriptional targets. This may be due to the transcriptional down regulation of essential co-factors Brf1,2 and Bdp1 (Figure 6E). Polr3g expression can nonetheless inhibit aspects of FGF/Wnt induced cell differentiation resulting in the decrease of skeletal muscle specific mRNAs.

## Discussion

Tissue specific expression of transcription factors is a common theme in developmental biology, where families of transcriptional regulators (eg, the bHLH myogenic regulatory factors) drive cell fate determination and differentiation. However, the restricted pattern of expression of basal transcriptional machinery, specifically the Polr3g and Polr3gL subunits of RNA polymerase III, during embryonic development is a novel finding. The documented role for the Polr3g-containing isoform of Pol III in proliferative, pluripotent cells (Wong et al., 2011; Haurie et al., 2010) suggests that the localised expression we show in embryos points to Polr3g expression in progenitor cells. The transient nature of Polr3g expression, from paraxial mesoderm and somite (early skeletal muscle lineage) to pronephric tubules (early kidney lineage) is consistent with its regulation during cell fate progression. The overall levels of Polr3g transcripts and protein are low subsequent to the early cleavage stages that are characterised by rapid cell division; however, western blotting and RT-PCR show that it is not entirely absent, and *in situ* hybridization analysis reveal highly regulated expression of Polr3g during development. The alternative isoform, Polr3gL is transcribed only zygotically and its expression is enriched in the developing nervous system, including the otic vesicle (developing ear) and branchial arches (derived from neural crest), as well as the ventral blood island. Its later expression in the differentiating somites is consistent with our findings that Polr3gL is expressed in response to myogenic induction in an explant model. Overexpression of Polr3gL results in downregulation of Pol III transcription in dorsal regions and upregulation of skeletal muscle specific genes, similar to the effects of myogenic induction. In contrast, Polr3g overexpression had some ability to reverse differentiation. The lack of overlap of the timing and the tissue specific expression of Polr3g isoforms indicates these genes are differentially regulated during development, this, together with their distinct activities suggest different, possibly sequential roles in myogenic determination and differentiation.

### *Analysing RNA polymerase III transcription in Xenopus*

To investigate any differential activity of Polr3g isoforms we analysed Pol III transcription in overexpression assays; our attempts at knockdown and gene targeting were unsuccessful due to the large amount of maternally deposited Polr3g mRNA and protein present in early embryos. Another consideration when considering this work is the large expansion of tRNA genes in *Xenopus*; the repetitive nature means that identifying any change in expression of any individual tRNA gene is not possible. By using a custom tRNA microarray we have been able learn about the transcriptional activity of sets of tRNA isoacceptors and isotypes. An alternative approach would be RNaseq following the methods of (Zheng et al., 2015) that uses methylase treatment to improve cDNA synthesis of tRNAs. This method disrupts tRNA structure to allow access to the polymerase during first strand synthesis for cDNA production, which make high throughput sequencing protocols possible for tRNA.



We found that Polr3g activity does not lead to uniform upregulation of Pol III targets, as may have been predicted from the presence of Polr3g in transformed cells (Haurie et al., 2010) and the upregulation of Pol III activity in cancer cells (Khattar et al., 2016). Instead, we find restricted sets of tRNA isoacceptors that are either up- or down-regulated at the start of transcription (Figure 4) or differentially effected at two different stages. For example, Polr3g overexpression upregulates tRNA<sup>Ala</sup> and tRNA<sup>Leu</sup> families at NF Stage 9 but not at NF Stage 25. The ability to activate or repress different target genes at different stages and regions suggests that Polr3g may have different transcriptional activity during development. These changes in Pol III transcriptional activity can result from interactions with co-factors or the influence of signal transduction pathways (reviewed (White and Sharrocks, 2010)). Polr3g is known to interact with Polr3C(RPC62) (Boissier et al., 2015) as part of a ternary complex with Polr3F (RPC39) essential for activating of transcription of target genes (Wang and Roeder, 1997). RNA Polymerase III is regulated by a number of developmental signalling pathways (Erk, TOR) and cellular regulators (p53, Rb, Myc, Maf1) which alters its activity *in vitro* (Felton-Edkins et al., 2003). It is well documented that dynamic signalling during development drives cell specification and determination by regulating Pol II activity, and we suggest here that Pol III activity is also disposed to regulation by cell signalling, providing an explanation for the differential effects of Polr3g activity in blastula and later stages of development.

*Some RNA Polymerase II targets are upregulated in blastula embryos overexpressing Polr3g.*

It is surprising that overexpression of Polr3g results in the selected upregulation of a few RNA Polymerase II regulated genes with roles in early development (Figure 4C). While other studies have indicated that Polr3g overexpression can upregulate RNA Polymerase II genes involved in pluripotency, this has been attributed to an indirect effect of Polr3g expression enhancing resistance to differentiation programmes in ESCs (Wong et al., 2011), which is not the case in these early embryos. We suggest an alternative mechanism, the shifting of the MBT, to explain the observation that Polr3g overexpression results in changes to RNA Polymerase II target genes in early *Xenopus* embryos. The genes identified as upregulated in embryos overexpressing Polr3g are early developmental regulators, normally transcribed just after MBT. MBT is preceded by rapid synchronous cell divisions known as cleavage during which embryos do not increase in size and, as a consequence, cells become smaller through consecutive divisions (Newport and Kirschner, 1982a). Previous studies in *Xenopus* have shown that MBT is triggered by the increase in Nuclear : Cytoplasmic (N:C) ratio; this mechanism is explained by the titration of limiting replication factors that are diluted during the rapid cell divisions and when reduced to a sufficient level, the cell cycle slows and zygotic transcription begins (Collart et al., 2013), (Newport and Kirschner, 1982a). It is possible that overexpression of Polr3g in *Xenopus* results in increased rates of cell division, raising the N:C ratio, and allowing the precocious activation of transcription of zygotic transcripts as MBT is brought forward (Figure 4). Cell culture studies have indicated that Polr3g increases cell proliferation rates in cancer cell lines and increased tumour growth (Khattar et al., 2016). In human ESCs, Polr3g expression results in increased levels of cell cycle regulators Aurora Kinase A and Cyclin E and the maintenance of pluripotency factors (Haurie et al., 2010), (Wong et al., 2011).



*Polr3g expression in the myogenic lineage*

We demonstrate here for the first time that different isoforms of RNA polymerase III are present in distinct cell lineages during vertebrate development. The specific expression of *Polr3g* in early somite cells likely marks a transient population of proliferative cells at an early stage during lineage determination. Our FGF/Wnt induction of myogenesis did not capture this time point, but instead a later point after the onset of expression of *Polr3gL* and other early differentiation markers (eg. *myh*, *actc*). Overall the ability of *Polr3g* to increase Pol III activity, together with its known presence in proliferative cells including *Xenopus* oocytes and pre-MBT embryos, suggests a role in augmenting levels of Pol III transcripts, ensuring sufficient tRNAs and other small RNAs as cell numbers increase. The specific yet transient expression of *Polr3g* in the early somites points to a progenitor population, still proliferative yet committed to the skeletal muscle cell lineage. *Polr3gL* is expressed during myogenic differentiation (and in other differentiating lineages), and its expression is associated with the decrease in Pol III activity, as seen in our explant study. It is worth noting that the pluripotency markers *Sox2* and *Oct4* are upregulated in explant model of myogenesis (Figure 6 D). This is not surprising given that the dorsal mesoderm induced by Wnt/FGF itself induces neural tissue which expresses both *Sox2* and *Oct4*, therefore it is not possible to draw any conclusions from this experiment in terms of pluripotency.

Nevertheless, we found a remarkable association of decreased Pol III activity during differentiation that could not be reversed by overexpressing *Polr3g*. This progressive down-regulation of Pol III transcription is also evident when comparing endogenous levels of Pol III transcripts at blastula stages with that of tailbud explants (supplemental data). Downregulation of Pol III activity has been observed previously during the differentiation of F9 carcinoma cells and was shown to be due to reduced levels of the TFIIB subunits Brf1 and Bdp1 (Athineos et al., 2010; White et al., 1989). Here, we extend candidates involved in the mechanism downregulating Pol III activity to much of its transcriptional machinery. The overexpression of *Polr3g* alongside *Fgf4*/Wnt8 in animal caps was not sufficient to reverse the downregulation of Pol III tRNA targets upon myogenic differentiation. This may be due to the high expression of *Polr3gL* at this stage competing with and overcoming *Polr3g* occupancy at target gene promoters. However, *Polr3g* expression did result in a significant reduction in the activation of muscle genes suggesting that, as in ESCs (Wong et al, 2011), *Polr3g* is able to increase resistance of cells to fully differentiate in the presence of inducing signals.

It is interesting to note recent findings that demonstrate Pol III regulation of microRNA transcription by a C/EBP $\beta$ -mediated activation of a tRNA-like enhancer element in miR-138 (Di Pascale et al., 2018). Since C/EBP $\beta$  restricts myogenic differentiation in satellite cells (Lala-Tabbert et al., 2016), and it is well known that MyomiRs are important players in myogenesis (Horak et al., 2016), these types of interactions could underlie our observed differential effects of Pol III isoforms on Pol II transcription. Overall, our findings attest to the importance of considering the activity of RNA polymerase III when building gene regulatory networks to describe myogenic differentiation.

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## Figure Legends

**Figure 1 Temporal expression analysis of *Polr3g* and *Polr3gL* expression during *Xenopus tropicalis* development.** (A) RT-PCR analysis of *Polr3g* and *Polr3gL* mRNA expression across a stage series of *X.tropicalis* development. 28S rRNA was used as an endogenous control. (B) Western blotting of protein extracts from a stage series of *X.tropicalis* embryos using an antibody generated against *X.tropicalis* *Polr3g*. GAPDH was used as a loading control. (C) Meta analysis of RNA-Seq data for *Polr3g* and *Polr3gL* across *Xenopus* development carried out by Owens et al. (2016) and deposited online via *Xenbase.org*.

**Figure 2 Expression of *Polr3g* in *Xenopus tropicalis* embryos.** Expression analysis of *Polr3g* *Xenopus tropicalis* at NF stage 20. (A) Lateral view of NF St20 embryo analysed by *in situ* hybridization for *Polr3g*; expression in the somites is indicated; anterior is to the left. (B) qRT-PCR for *Polr3g* expression in the early tailbud (NF St23). Head, Ventral and Dorsal sections were dissected and RNA extracted for qPCR. Ct values were normalised to the gene *Dicer*. All expression values were calculated relative to somite expression. One-way ANOVA statistical analysis was carried out on relative expression of each section for each gene (\*) represents the level of significance. (C) Dorsal view of NF St20 embryo analysed by *in situ* hybridization for *Polr3g*; a cross-section through the embryo shown in (D) is indicated; anterior is to the left. (D) Cross section of embryo shown in (C) shows specific expression of *Polr3g* in somites at NF St20.

**Figure 3 Expression of *Polr3gL* in *Xenopus tropicalis* embryos.** Expression analysis of *Polr3gL* *Xenopus tropicalis* at NF stage 20. (A) Lateral view of NF St20 embryo analysed by *in situ* hybridization for *Polr3gL*; expression in the anterior neural tube (nt), otic vesicle (ov), and branchial arches (ba) is indicated; anterior is to the left. (B) qRT-PCR for *Polr3gL* expression in the early tailbud (NF St23). Head, Ventral and Dorsal sections were dissected and RNA extracted for qPCR. Ct values were normalised to the gene *Dicer*. All expression values were calculated relative to somite expression. One-way ANOVA statistical analysis was carried out on relative expression of each section for each gene (\*) represents the level of significance. (C) Lateral view of NF St28 embryo analysed by *in situ* hybridization for *Polr3gL*; expression in the otic vesicle (ov), and branchial arches (ba), blood island (bi), and posterior somites is indicated. A cross-section through the embryo shown in (D) is indicated; anterior is to the left. (D) Cross section of embryo shown in (C) shows specific expression of *Polr3g* in posterior somites at NF St28.

**Figure 4 Changes in tRNA expression in response to *Polr3g* overexpression.** Expression fold change summary of tRNA isoacceptor families in *X.tropicalis* NF Stage 9 whole embryos injected with 2ng *Polr3g* mRNA compared to control uninjected stage 9 embryos across 3 biological replicates. Signal data was processed using GeneSpring to determine normalized signal values for each probe within each array, and a normalized intensity reading by comparing probes across the arrays; these values were used to determine expression fold changes for each biological replicate to give an average fold change for each probe across the three replicates. Control and *Polr3g* injected mean expressions for each of the 5 probes designed per isoacceptor family were then analysed using paired t-tests to determine statistical significance of the change in expression for each anticodon isoacceptor family. To account for multiple comparisons, Bonferroni Correction was applied. (A) Summary chart of fold changes for isoacceptor families with raw signals of >10. Orange indicates significantly up-regulated

transcription, blue indicates significantly down-regulated transcription, and pink indicates no significant change in expression. SEM is presented as error bars and \* indicates the level of statistical significance. (B) Summary chart of fold changes for other Pol III target genes with raw signals of >10. (C) Summary chart of fold changes for mRNAs upregulated in Polr3g overexpression samples.

**Figure 5 Overexpression of Polr3g compared to that of Polr3gL in *Xenopus tropicalis* embryos.**

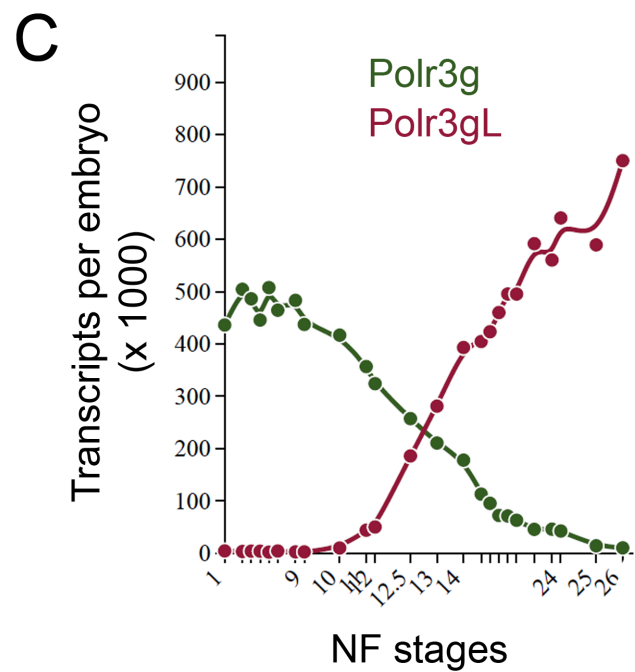
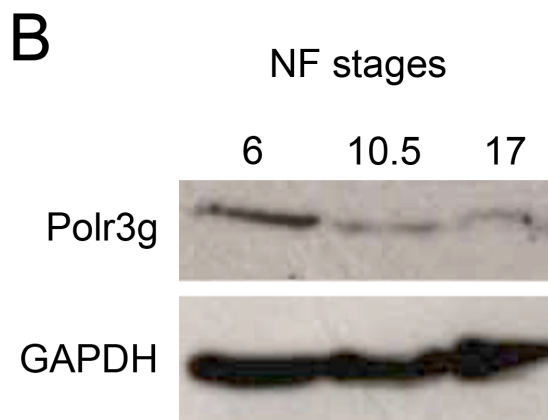
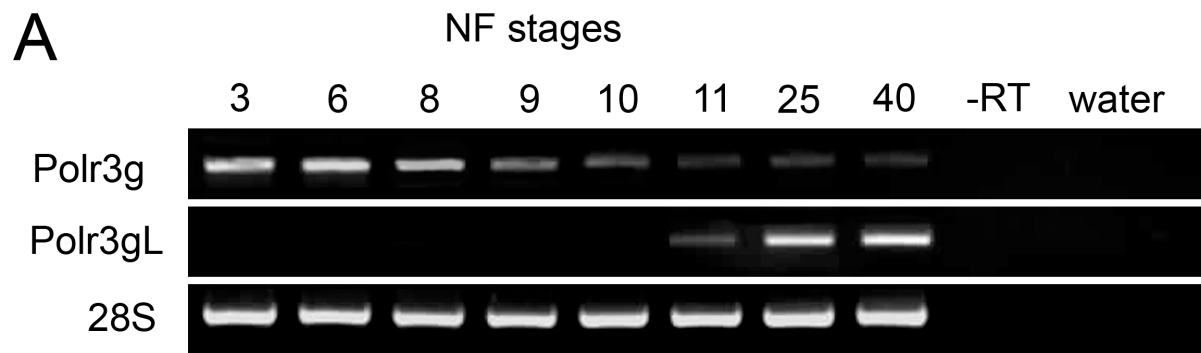
(A) 2ng of mRNA coding for HA-tagged Polr3g and Polr3gL was injected into both blastomeres of *X.tropicalis* embryos at the 2-cell stage and embryos were allowed to develop until NF Stage 25. Western blot analysis of injected and control embryos for detection of HA-tag expression shows equal levels of protein expression in injected embryos. GAPDH was used as a loading control. (B) Dorsal regions were dissected to enrich for myogenic tissue at this stage of development (labelled here by *in situ* hybridization for *MyoD*). (C) Expression fold change summary of tRNA isoacceptor families in *X.tropicalis* NF Stage 25 dorsal sections injected with 2ng Polr3g mRNA versus control uninjected across 3 biological replicates. (D) Expression fold change summary of tRNA isoacceptor families in *X.tropicalis* NF Stage 25 dorsal sections injected with 2ng Polr3gL mRNA versus control uninjected across 3 biological replicates. Orange indicates up-regulated transcription, blue indicates down-regulated transcription, and pink indicates no change in expression. SEM is presented as error bars and \* indicates the level of statistical significance between control and injected samples after Bonferroni Correction. (E) Scatterplot comparing normalised expression of tRNA isoacceptor families in embryos overexpressing Polr3g compared to those overexpressing Polr3gL. Solid line indicates no expression difference. Dashed lines indicate 2 fold change in expression. Polr3g values are on the X-axis and Polr3gL values are on the Y-axis. (F) Expression fold change summary of selected contractile proteins in *X.tropicalis* NF Stage 25 dorsal sections injected with 2ng of Polr3g (orange) or Polr3gL (blue) mRNA versus control uninjected across 3 biological replicates. SEM is presented as error bars and \* indicates the level of statistical significance between control and injected samples after Bonferroni Correction.

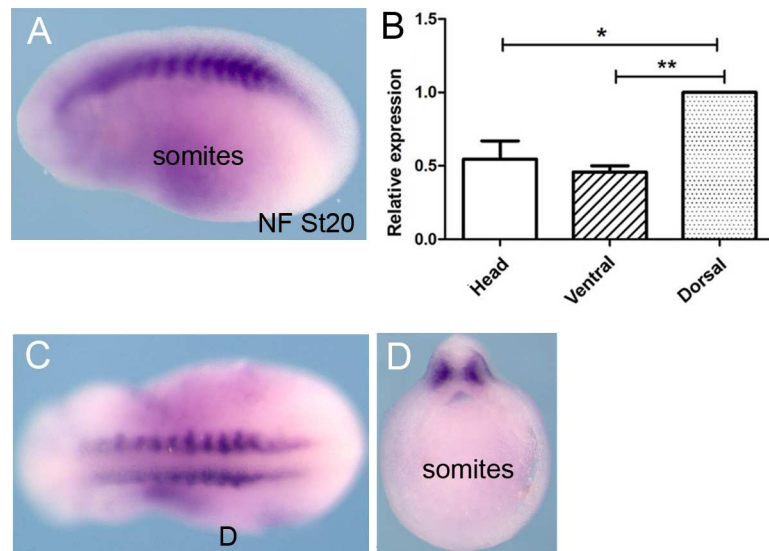
**Figure 6. Downregulation of Pol III activity in a myogenic explant model.** Overexpression of Fgf4 + Wnt8 in NF Stage 25 *X.tropicalis* animal explants results in a downregulation of RNA Polymerase III transcripts and an upregulation of mRNAs associated with myogenic differentiation. Expression fold change summary of *X.tropicalis* NF Stage 25 animal cap explants dissected from embryos injected with 5pg Fgf4 and 50pg Wnt8 mRNA compared to explants taken from control uninjected embryos across 3 biological replicates. Only RNAs with raw signal intensities of >10 were included in the analyses. (A) RNA Polymerase III target genes (significantly altered tRNA isoacceptor families are in blue, other significantly altered RNA Polymerase III targets are shown in red). (B) Contractile protein gene expression is highly upregulated (note the log scale). (C) Expression of the Myogenic Regulatory Factor (MRF) genes is also upregulated in Fgf4 + Wnt8 explants. (D) Selected genes associated with pluripotency and cell cycle regulators are differentially affected in Fgf4 + Wnt8 explants. (E) Overexpression of Fgf4 + Wnt8 in NF Stage 25 animal cap explants results in downregulation of some genes coding for proteins associated with RNA Polymerase III core transcription machinery. Pink in all panels indicates no significant change in expression, while blue is significantly down and orange is significantly up. SEM is presented as error bars and \* indicates the level of statistical significance between control and injected samples after Bonferroni Correction.

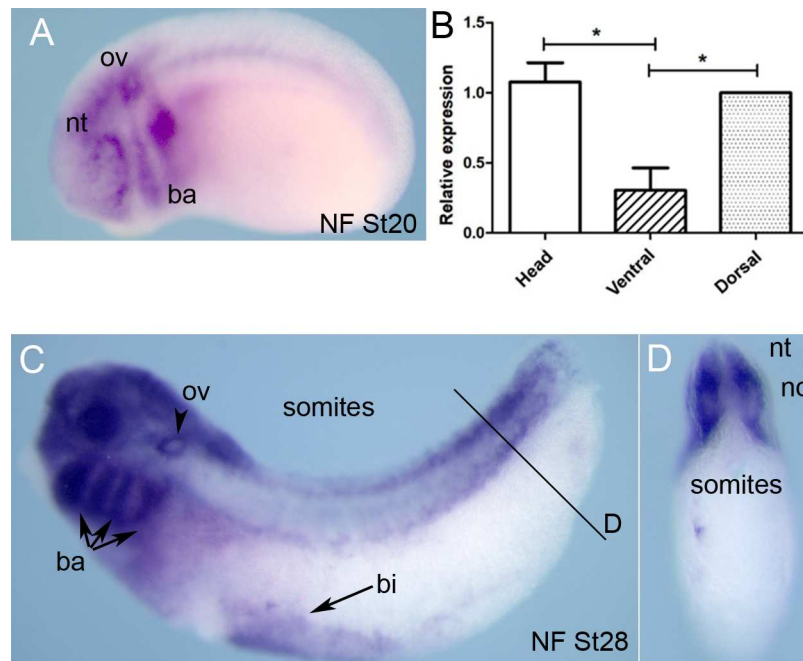
**Figure 7 Polr3g overexpression restricts myogenic differentiation in an explant model of myogenesis without eliciting any recovery of tRNA expression**

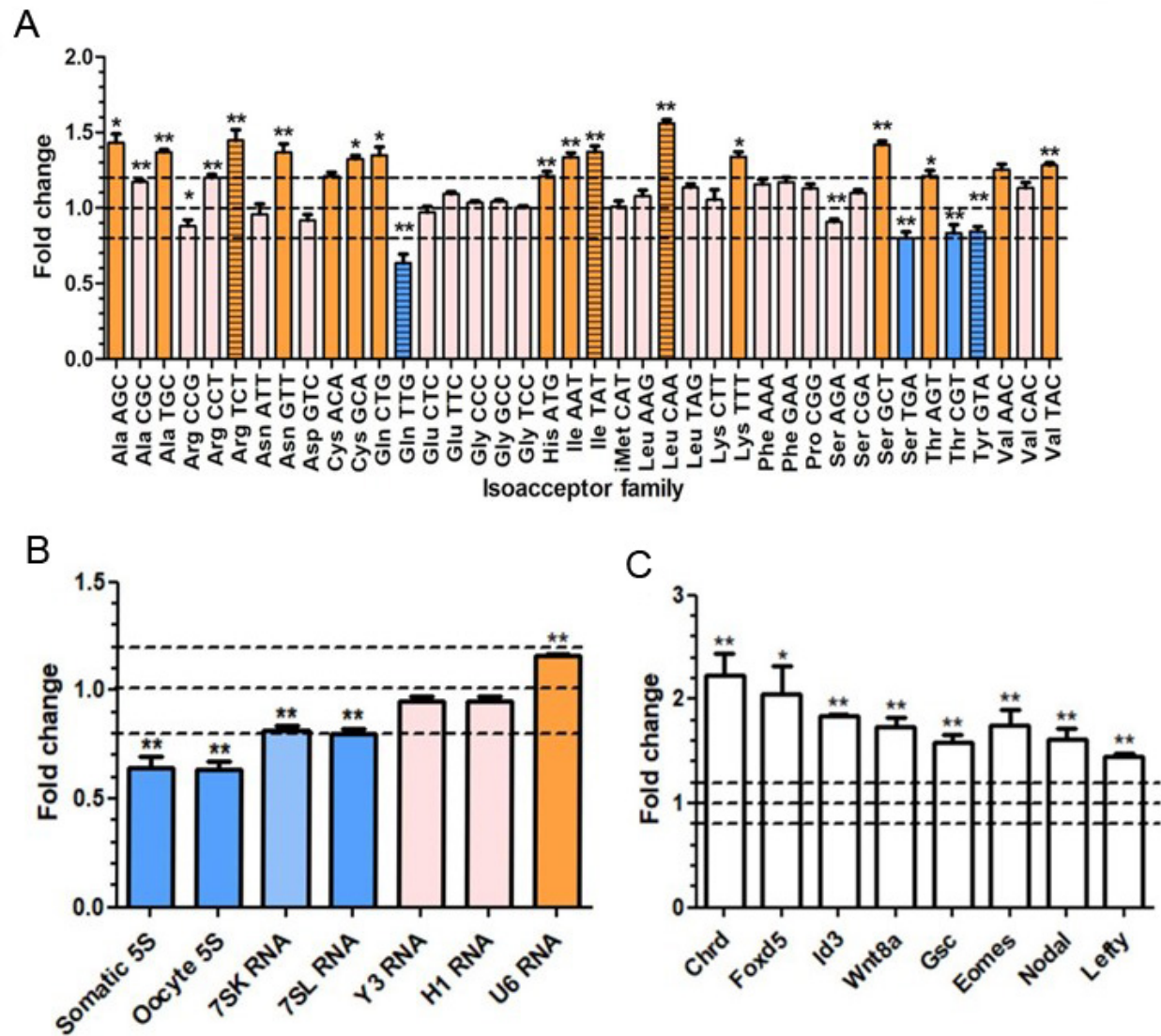
qRT-PCR analysis of RNA extracted from explants injected with either Fgf4 + Wnt8, or Fgf4+ Wnt8 + Polr3g and cultured to stage 25. **(A)** Analysis shows a significant decrease in tRNA isoacceptors for iMethionine<sup>CAT</sup>, Tyrosine<sup>GTA</sup> and Alanine<sup>AGC</sup> in both experimental groups as compared to uninjected control explants at stage 25. Pair-wise *t*-tests were carried out for each tRNA's mean relative expression comparing the mean Ct values normalised to control gene *Dicer*, for the three biological replicates, between the uninjected controls and the experimental groups. Error bars represent SEM. **(B)** qPCR analysis shows high increase in differentiation specific mRNAs *actc1*, *act3* and *myh4* activated by myogenic inducing factors FGF4+Wnt8. The mean relative expression of two biological replicates was plotted (muscle gene expression is not detectable in un-induced animal caps). Mean fold changes of 3,306 (*actc1*), 249,870 (*act3*) and 96,436 (*myh4*) were found in Fgf4/Wnt8 samples and set to 1.0; these were reduced to 14%, 7% and 15% of this level, respectively, when Polr3g was co-expressed. Error bars represent SEM.



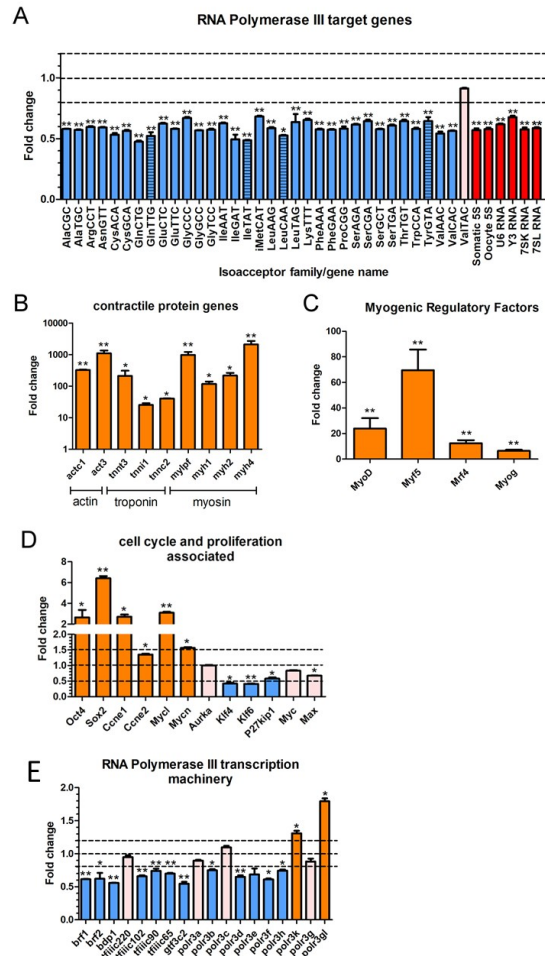




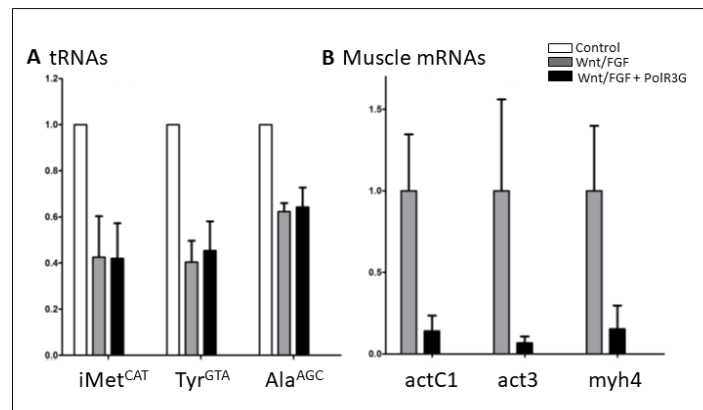












**Highlights:**

- There are 2 distinct isoforms of Pol III with either the subunit Polr3g or Polr3gL
- Polr3g and Polr3gL have complementary expression during development
- Polr3g is transiently expressed in early somites; Polr3gL is activated later
- Pol III activity decreases during differentiation, while Polr3gL increases
- Expression of Polr3g is not compatible with differentiation